D. N. 0203.00

Title: *PiggyBac* Transformation System

[0001] This application claims benefit to copending provisional application serial number 60/225,827, filed August 17, 2000; which is herein incorporated by reference.

BACKGROUND OF INVENTION

Field of the Invention

[0002] This invention relates to a transformation system that includes a gene transfer vector containing a modified *piggyBac* transposon (pB) and having the insertion of a marker construct containing at least one fluorescent protein gene linked to a polyubiquitin promoter gene. The invention further relates to a helper vector containing a heat shock protein gene and to methods for using this system to transform eukaryotic cells as well as transgenic organisms produced using the system, especially insect cells and insects, respectively.

Description of the Related Art

[0003] The *piggyBac* transposable element from the cabbage looper moth, *Trichoplusia ni* (Cary et al., Virology, Volume 161, 8-17, 1989) has been shown to be an effective gene-transfer vector in the Mediterranean fruit fly, *Ceratitis capitata*

(Handler et al., Proc. Natl. Acad. Sci. USA, Volume 95, 7520-7525, 1998). Use of an unmodified transposase helper under *piggyBac* promoter regulation indicates that *piggyBac* retains autonomous function in the medfly, since transcriptional regulation was maintained, as well as enzymatic activity. This observation was unique since all other successful insect germline transformations had been limited to dipteran species using vectors isolated from the same or another dipteran. The initial transformation of medfly (Loukeris et al., Science, Volume 270, 2002-2005, 1995) used the *Minos* vector from *Drosophila hydei* (Franz & Savakis, Nucl. Acids Res., Volume 19, 6646, 1991), and *Aedes aegypti* has been transformed from *Hermes* (Jasinskiene et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3743-3747, 1998) from *Musca domestica* (Warren et al., Genet. Res. Camb., Volume 64, 87-97, 1994) and *mariner* (Coates et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3748-3751, 1998) from *Drosophila mauritiana* (Jacobson et al., Proc. Natl. Acad. Sci. USA, Volume 83, 8684-8688, 1986). *Drosophila melanogaster* has been transformed as well by *Hermes* (O'Brochta et al., Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996) *mariner* (Lidholm et al., Genetics, Volume 134, 859-868, 1993), *Minos* (Franz et al., Proc. Natl. Acad. Sci. USA, Volume 91, 4746-4750, 1994) and by the *P* and *hobo* transposons

originally discovered in its own genome (Rubin and Spradling, 1989; Blackman et al., EMBO J., Volume 8, 211-217, 1989). *Drosophila virilis* also has been transformed by *hobo* (Lozovskaya et al., Genetics, Volume 143, 365-374, 1995; Gomez & Handler, Insect Mol. Biol., Volume 6, 1-8, 1997) and *mariner* (Lohe et al., Genetics, Volume 143, 365-374, 1996). While the restriction to dipteran vectors is due in part to the limited number of transposon systems available from non-dipteran species, phylogenetic limitations on transposon function is not unexpected considering the deleterious effects functional transposons may have on a host genome. This is, indeed, reflected by the high level of regulation placed on transposon movement among species, among strains within a host species, and even among cell types within an organism (Berg & Howe, *Mobile DNA*, American Society for Microbiology, Washington, D.C. 1989).

[0004] The ability of *piggyBac* to function in several dipteran species will be supportive of its use in a wider range of insects, if not other organisms. Most other vector systems function optimally, or have been only tested with their helper transposase under *hsp*70 promoter regulation. The transposition efficiency of most vectors has been also found to be influenced by the amount of internal DNA inserted, the position of this DNA

within the vector, and the amount of subterminal DNA remaining in the vectors.

[0005] The widespread use of *piggyBac* will be limited by the availability of easily detectable and unambiguous transformant markers. Most Drosophila transformations, as well as the few nondrosophilid transformations reported have depended on transformant selection by rescue of a mutant visible phenotype, usually eye pigmentation (Ashburner et al., Insect Mol. Biol., Volume 7, 201-213, 1998). Unfortunately, most insect species have neither visible mutant strains, nor the cloned DNA for the wild type allele of the mutation, and these species require use of new dominant-acting marker genes that confer, preferably, a visible phenotype.

[0006] The present invention, discussed below, provides a system that includes vectors for transforming eukaryotic cells, derived from *piggyBac* transposons that are different from related art vectors. Furthermore, the present invention increases the transformation frequency by about eight-fold compared to other *piggyBac* transformation systems.

Summary of the Invention

[0007] It is therefore an object of the present invention to

provide a transformation system which contains a vector that includes DNA derived from a *piggyBac* transposon element that allows for the almost precise excision of at least a second DNA sequence that is heterologous and included in the construct and insertion of at least said second heterologous DNA sequence into eukaryotic cells after introduction of the transformation construct containing said first and at least a second DNA into said cell that is then used to form a transgenic organism wherein said transgenic organism is detectable under ultraviolet light.

[0008] Another object of the present invention is to provide a transformation system that includes a vector containing a modified *piggyBac* sequence, at least one sequence for marker expression linked to a polyubiquitin promoter and a helper vector including a heat shock protein gene wherein said system causes an increase in transformation frequency compared to other *piggyBac* transformation systems.

[0009] Another object of the present invention is to provide a transgenic organism that is detectable under ultraviolet light.

[0010] A further object of the present invention is to provide a transformation system that includes a vector containing a modified *piggyBac* sequence, at least one fluorescent protein gene

linked to a polyubiquitin promoter, and a helper vector including a heat shock protein gene.

[0011] Further objects and advantages of the present invention will become apparent from the following description.

Brief Description of the Drawings

[0012] Figure 1(a) is a photograph of eye color phenotypes of D*m*[pB*w*] transformants.

[0013] Figure 1(b) is a photograph of a w[m] host strain fly (top) and orange-eye D*m*[pB*w*,*gfp*] transformant fly (bottom) under brightfield (left) and ultraviolet light (right).

[0014] Figure 1 (c) is a photograph of a *w*[m] host strain fly (top) and white-eye D*m*[pB*w*, gfp] transformant fly (bottom) under bright field (left) and ultraviolet light (right).

[0015] Figure 2 (a) is a schematic (not to scale) of the pB[Dm*w*] vector showing the *Bgl*II, *Sal*I, and *Nsi*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded gray, and the mini-*white* marker gene is white.

[0016] Figure 2 (b) shows an autoradiogram of a Southern DNA hybridization analysis of D*m*[pB*w*] transformant sublines, and w[m] host strain control samples from transformants using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers using *Bgl*II digestion and *Sph/Hpa piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

[0017] Figure 2(c) shows a Southern DNA hybridization analysis of D*m*[pB*w*] transformant sublines and *w*[m] host strain control samples from transformants, using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers ,using *Sal*I digestion and *Hpa/Ase piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

[0018] Figure 2 (d) shows a Southern DNA hybridization analysis of D*m*[pB*w*] transformant sublines and *w*[m] host strain control samples from transformants, using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers, using *Nsi*I digestion and *Nsi/Hpa* + *Hpa/Nsi* probes. DNA size markers are shown to the left

of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

[0019] Figure 3(a) is a schematic (not to scale) of the pB[Dmw, PUb-nls-EGFP] vector showing the *Bgl*II, *Xho*I, and *Pst*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). The *Sph/Hpa piggyBac* as probe contains 0.67 kb of vector sequence (*Sph*I to *Bgl*II) with *Bgl*II to *Hpa*I *piggyBac* sequence deleted from the vector. Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded gray, the mini-*white* marker gene is white, and the EGFP marker gene is hatched.

[0020] Figure 3(b) is an autoradiogram of a Southern DNA hybridization analysis of D*m*[pB*w*, *gfp*] transformant sublines, and wild type (wt) and *w*[m] host strain control samples using *Bgl*II digestion and *Sph/Hpa piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines with selected G1 transformant progeny of samples.

[0021] Figure 3 (c) is an autoradiogram of a Southern DNA hybridization analysis of D*m*[pB*w*, *gfp*] transformant sublines, and

wild type (wt) and *w*[m] host strain control samples using *Xho*I digestion and *Hpa/Ase piggyBac* fragment as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with selected G1 transformant progeny of samples.

[0022] Figure 3 (d) is an autoradiogram of a Southern DNA hybridization analysis of D*m*[pB*w*, *gfp*] transformant sublines, and wild type (wt) and *w*[m] host strain control samples using *Pst*I digestion and *Hpa/Ase piggyBac* fragment + EGFP DNA as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with specific G1 line numbers are given below, with the designation (+) for those expressing visible eye pigmentation and (-) for those having non-pigmented white eyes.

[0023] Figures 4 (a) and (b) show inverse PCR strategy to isolate the pB[Dm*w*] vector insertion site in transformant sublines. Figure 4 (a) is a schematic (not to scale) of the vector insertion in the host plasmid showing the approximate location of the restriction sites and primers used for PCR. Forward (F) and reverse (R) primers are numbered according to their nucleotide position in *piggyBac*. The *piggyBac* sequence is shown in gray surrounded by the TTAA (SEQ ID NO 1) duplicated

insertion site, the mini-*white* marker gene is white, and chromosomal sequence is hatched.

[0024] Figure 4(b) shows the *piggyBac* insertion site sequence in p3E1.2 (SEQ ID NOs 7 and 8), and the proximal insertion site sequences (SEQ ID NOs 9 and 10, 11 and 12, and 13 and 14) for three of the transformant sublines.

[0025] Figure 5 shows a circular map of the vector pB[PUb-nls-EGFP] #257.

[0026] Figures 6a-6f show SEQ ID NO 6 for pB[PUb-nls-EGFP]#257.

[0027] Figure 7(a) is a photomicrograph showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUb-nls-EGFP (pB[PUb-nls-EGFP]) at embryo stages. Under ultraviolet light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

[0028] Figures 7(b) and 7(c) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with pB[PUb-nls-EGFP] at larval stages. 7(b) is a wild-type non-transformant and 7c is a transformant. Under ultraviolet light, transformants exhibit bright green fluorescence, with wild-type non-

transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

[0029] Figures 7(d) and 7(e) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with pB[PUb-nls-EGFP] at adult stages. 7(d) is a wild-type non-transformant and 7(e) is a transformant. Under ultraviolet light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

[0030] Figures 8(a)-8(e) are eye color phenotypes of *Bactrocera dorsalis* wild-type (+) and white eye (WE) host strain and the Bd[pBCcw] transformant lines 61,115, and 137.

[0031] Figures 9(a) and 9(b) show medfly, *Ceratitis capitata* transformed with *piggyBac*/white/EGFP vector (pB[Ccw,pUB-nls-EGFP]) expressing eye color under brightfield (9a) and GFP expression under ultraviolet light (9b).

[0032] Figure 10 shows a transgenic insect having three integrations observed under ultraviolet light after various times after decapitation. Flies were decapitated at day 0, taped in a plastic box placed outdoors in partial sunlight. Digital

photographs were taken each day at the same exposure and magnification.

[0033] Figure 11 shows a circular map of pB[PUbDsRed1].

[0034] Figure 12 is a photograph which shows a Drosophila melanogaster strain transformed with the pB[PUbDsRed1] vector (*piggyBac* marked with DsRed1 fluorescent protein gene) at adult (top), pupal (middle) and larval (bottom) stages. Images taken under ultraviolet light with a Texas red filter.

[0035] Figure 13 shows the expression of DsRed in Drosophila transformed with pB[PUbDsRed] under Brightfield (left) and epifluorescence optics with a Texas red filter (right). A transformant (bottom) is compared to a wild host (top).

0036] Figure 14 shows expression of DsRed from the bentral (left) and dorsal (right) view in a Caribbean fruit fly transformed with pB[PUbDsRed] under epifluorescence optics with a Texas red filter.

[0037] Figure 15 shows expression of DsRed in a Caribbean Fruit fly transformed with pB[PUbDsRed] under Brightfield (left) and epifluorescence optics with a Texas Red Filter (right). A transformant (bottom) is compared to a wild host (top).

Detailed Description of the Invention

[0038] The present invention is an effective transformation system for producing transgenic organisms, especially transgenic insects. The identification and isolation of an autonomous *piggyBac* transposon enables transformation of cells and the production of transgenic organisms wherein DNA capable of being expressed in the transformed cell or transgenic organism is excised from a transformation construct and inserted into the genome of a cell used to produce a transgenic organism (United States Patent 6,218,185, issued April 17, 2001; herein incorporated by reference). The term cell for the purposes of this invention includes any cell capable of being transformed by the transformation construct of the present invention, and preferably includes any eukaryotic cell. The term organism for the purposes of the present invention includes any unicellular or multicellular living entity capable of being transformed by the transformation construct of the present invention and preferably includes multicellular eukaryotes. More preferably, the cell or organism is an insect cell or an insect.

[0039] The present invention utilizes the transposon machinery of the TTAA (SEQ ID NO 1) specific transposons to excise and insert a targeted functional heterologous DNA sequence into the

genome of the host cell. The resulting transformed cell or group of cells are stable transformants that are then used to make a transgenic organism, using techniques known to the skilled artisan, that will pass the introduced gene to all subsequent progeny. The targeted functional heterologous DNA for purposes of this invention is any heterologous DNA capable of being expressed in a host cell and/or a transgenic organism.

[0040] The transformation system of the present invention includes a vector, such as, for example, pB[PUb-nls-EGFP], pB[PUbDsRed1], etc. (Figures 5, 6, and 11), that includes a modified *piggyBac* transposon (pB) and a marker construct that includes a fluorescent protein gene under the regulation of a polyubiquitin promoter region. Any fluorescent protein gene capable of being expressed in a transgenic organism is useful in the present invention. Examples of useful fluorescent protein genes are an enhanced green fluorescent protein gene (EGFP), red fluorescent protein gene (DsRed1), blue fluorescent protein gene (BFP), yellow fluorescent protein gene (YFP), cyano fluorescent protein gene (CFP), etc., linked to the promoter region of the *Drosophila melanogaster* polyubiquitin (PUb) gene and the nuclear localizing sequence (nls) of the SV40 virus. These vectors containing at least one fluorescent protein gene, can be used to

transform and detect transgenic organisms based on expression of the fluorescent protein marker under ultraviolet light. After chromosomal integration and inheritance of the vector, expression of the fluorescent protein occurs in all cell types and is intense. When the NLS vector is used the expression of the fluorescent protein is strongly localized to nuclei. The fluorescent protein continues to be detectable under ultraviolet light even after death of the organism (Figure 10). One of the novel features of this vector includes its construction that deletes about 748 bp of internal *piggyBac* sequence without diminishing its function, and the function of the polyubiquitin promoter in a nondrosophilid species. The vector was created by restriction endonuclease digestion of *piggyBac* within the p3E1.2 plasmid at the unique *Bgl*II site at position 3113 and the unique *Hpa*I site at position 3861. The PUb-nls-fluorescent protein marker cassette was then ligated into the 748 bp deleted region. This has utility as a broadly based method for the creation and selection of transgenic organisms, and as a genetic marker for detecting and tracking transgenic insects used in field release programs (Figure 5).

[0041] Fluorescent protein expressivity is useful for nondrosophilid species not amenable to mutant-rescue, it also

widens the possibility for using the dominant expression of flourescent protein as a primary transformant marker in many *Drosophila* lines not already carrying the *white* or *rosy* mutations, or for screens requiring selection in early development. Though vectors carrying *white* and *gfp* have been tested previously, the transformations used only white as the transformant selection, with GFP assessed secondarily for specific spatial or developmental expression (Davis et al., Devel. Biol., Volume 170, 726-729, 1995; Wang & Hazelrigg, Nature, Volume 369,400-403, 1994).

[0042] The transformation system of the present invention also includes a *piggyBac* transposase helper plasmid, pBΔSac, having its' 5' terminus deleted as described by Handler et al. (1998, *supra*; herein incorporated by reference). A new transposase helper under heat-shock promoter regulation was created by the isolation of the 457 bp *Xba*I-*Xmn*I 5' nontranslated sequence from the *hsp*70 gene (Lis et al., Cell, Volume 35, 403-410, 1983, herein incorporated by reference). The heat-shock regulated helper increases the transformation frequency by eight-fold in *Drosophila*, indicating that the *piggyBac* system could be as effective as routinely used systems such as *P* and *hobo* that have

been thus far inactive in nondrosophilids (O'Brochta & Atkinson, Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996).

[0043] The creation of a transformed cell requires that the vector containing the functional heterologous DNA first be physically placed within the host cell. Current transformation procedures utilize a variety of techniques to introduce DNA into a cell. In one form of transformation for vertebrate systems, the DNA is microinjected directly into embryos through the use of micropipettes. Alternatively, high velocity biolistics can be used to propel small DNA associated particles into the cell. In another form, the cell is permeablized by the presence of polyethylene glycol, thus allowing DNA to enter the cell through diffusion. DNA can also be introduced into a cell by fusing protoplasts with other entities that contain DNA. These entities include minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Electroporation is also an accepted method for introducing DNA into a cell. In this technique, cells are subject to electrical impulses of high field strength that reversibly permeabilizes biomembranes, allowing the entry of exogenous DNA sequences. One method of introducing the transformation system of the present invention into insect embryos, in accordance with the present invention, is to

microinject fertilized eggs with the vectors of the present invention. The DNA sequence flanked by the transposon inverted repeats will be inserted into the genome of some of the germ cells of the fertilized egg during development of the organism. This DNA will then be passed on to all of the progeny cells to produce transgenic organisms. The microinjection of eggs to produce transgenic animals has been previously described and utilized to produce transformed mammals and insects (Rubin et al., Science, Volume 218, 384-393, 1982; Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1986; Morgan et al., Annu. Rev. Biochem., Volume 62, 191-217, 1993; Spradling, A.C., In: Drosophila: A Practical Approach, ed. D.B. Roberts, Oxford: IRL Press, 175-197, 1986; all herein incorporated by reference). Accordingly, a method of producing stably transformed insects includes the step of microinjecting the transformation constructs of the present invention comprising the inverted repeats of a TTAA (SEQ ID NO 1) specific transposon and a helper construct into a cell, preferably a fertile insect egg. This is followed by incubation in an oxygenated and humidified tissue culture chamber at about 22-23° C for about 3-6 hours. Injected cells or eggs are then heat shocked at about 37°-41° C, about 39°C

preferred, for about 1 hour. The resulting transformed cells or transgenic organisms have exogenous DNA inserted into the genomic DNA at the sequence TTAA (SEQ ID NO 1).

[0044] Transformed cells and/or transgenic organisms can be selected from untransformed cells and/or non-transgenic organisms by ultraviolet light since the transformation system includes at least one fluorescent protein gene that produces an altered visible phenotype under ultraviolet light. Using standard techniques known to those familiar with the field, techniques such as, for example, Southern blotting and polymerase chain reaction, DNA can be isolated from transformed cells and/or transgenic insects to confirm that the introduced DNA has been inserted.

[0045] Genetic modification of insects with new genetic elements provides a means to control populations of agriculturally pestiferous or beneficial insects. The ability to control pest insects through genetically based sterile insect programs or genetically introduced targeted conditional susceptibilities will result in significant cost savings to agribusiness. This technology can also be used for detection and monitoring of insect populations and infestations where *piggyBac* transgenic insects are present in the population. In addition,

introduction of genes that impart resistance to chemicals (including herbicides, pesticides, and insecticides) can improve the efficacy of beneficial insects. Each of these applications will result in more efficient pest control programs.

[0046] Enhancing the resistance of beneficial insects to pesticides will enhance the efficacy of the beneficial insects and may allow for the simultaneous use of chemical control and biological control of pests. Some of the beneficial insects that would make good candidates for such transformations include Hymenopteran parasitoids of Heliothis spp.: *Micropilitis croceips* and *Cardiochiles nigriceps*; Hymenopteran parasitoid of Diamondback moth, *Plutella xylostella*: *Diadegma insolare*; Hymenopteran parasitod of the Indianmeal moth, *Plodia interpunctella*: *Bracon hebitor*; and Hemipteran predators: *Xylocoris flavipes*, *Podisus maculatus*.

[0047] The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention as described by the claims.

EXAMPLE 1

[0048] The *piggyBac* transposase helper plasmid, pBΔSac,

having its 5' terminus deleted was described previously (Handler et al., 1998; *supra*, herein incorporated by reference). pBΔSac was created by digestion of p3E1.2 (United States Patent Application 08/844,274) with *Sac*I and religation, that deletes the 5' *piggyBac* terminal sequences but maintains the putative *piggyBac* promoter region. A transposase helper under heat-shock promoter regulation was created by isolation of the 457 bp *Xba*I-*Xmn*I 5' nontranslated sequence from the *hsp*70 gene (Lis et al., 1983, *supra*; herein incorporated by reference). The *Xba*I-*Xmn*I fragment was blunted and ligated into the *Sac*I-blunted site of pBΔSac to create phsp-pBac. This places the *hsp*70 promoter sequence upstream of the putative *piggyBac* promoter.

[0049] The pB[Dmw] vector was created by insertion of a *Drosophila melanogaster* mini-*white* gene (Pirrotta et al., EMBO J., Volume 4, 3501-3508, 1985; herein incorporated by reference) into the 3E1 *piggyBac* element within the 6.0 kb p3E1.2 plasmid (Cary et al., 1989, *supra*). The mini-*white* gene was isolated as a 4.2 kb *Eco*RI fragment, blunted and ligated into the p3E1.2 *Hpa*I site. The inserted *w* gene interrupts the *piggyBac* open reading frame (ORF), but otherwise leaves the *piggyBac* element intact, with the respective promoters in opposite orientation. A

piggyBac vector marked with *w* and *gfp* was created by initial construction of *piggyBac* marked with an enhanced *gfp* regulated by *D. melanogaster* polyubiquitin (PUb) promoter (Lee et al., Mol. Cell. Biol., Volume 8, 4727-4735, 1988; herein incorporated by reference) linked in-frame to the SV40 nuclear localizing sequence (nls) (Lanford et al., Mol. Cell. Biol., Volume 8, 2722-2729, 1986). The polyubiquitin-nls (PUb-nls) cassette from PUbnlsGFP (Davis et al., 1995, *supra*) was isolated as *Kpn*I-*Sma*I fragment and inserted into the *Kpn*I-*Sma*I cloning site of EGFP-1 (Clontech) (Cormack et al., Gene, Volume 173, 33-38, 1996; Yang et al., Nucleic Acid Res., Volume 24, 4592-4593, 1996). Polyubiquitin-nls-EGFP was then isolated as a 4.1 kb *Bgl*II-*Stu*I fragment and ligated into the *Bgl*II-*Hpa*I site of *piggyBac* within p3E1.2 to create pB[PUb-nls-EGFP]. The *Bgl*II-*Hpa*I digestion results in a 748 bp deletion within p3E1.2. The mini-*white* gene was then inserted into the unique *Bgl*II site by blunt-end cloning to create pB[Dm*w*, PUb-nls-EGFP].

EXAMPLE 2

[0050] Embryo injections used standard procedures (Rubin & Spradling, Science, Volume 218, 348-353, 1982; herein incorporated by reference) with dechorionation achieved either

manually or by 1.6% hypochlorite solution followed by about 2 washings in approximately 0.02% Triton-X 100 in water. Eggs were placed on double-stick tape, desiccated in room-air for about 10-15 minutes and submerged under Halocarbon 700 oil. Injections followed standard *Drosophila* microinjection procedures (Rubin and Spradling, Science, Volume 218, 348-353, 1982; herein incorporated by reference). DNA mixtures had vector:helper concentrations of about 600:400 µg/ml, respectively, in injection buffer (approximately 5 mM KCl; approximately 0.1mM sodium phosphate; at about pH 6.8). Injected eggs were placed in an oxygenated and humidified tissue culture chamber at about 22-23°C for about 3-6 hours, and phsp-pBac injected eggs were heat shocked at about 37°C for about one hour. Hatched larvae were collected about 1-2 days later and placed on larval diet. Eclosed G0 male adults were mated either individually to about 2 or 3 *w*[m] virgin female adults, or in groups of about three females to about six males. G1 eggs were collected for two weeks and reared under standard conditions that include maintaining the eggs at about 23-25° C on standard cornmeal-yeast-molasses media (Ashburner et al., *supra*).

[0051] Green fluorescent protein (GFP) was observed at all developmental stages under a Leica MZ-12 stereozoom microscope

using a mercury lamp and an epifluorescence longpass filter set (HQ 41012 FITC; Chroma) optimized for red-shifted GFP variants. Photographic documentation used an Olympus OM-4 camera and 400 ASA Fujichrome film with exposure times that were determined empirically.

[0052] In the first of three transformation experiments, the *piggyBac* vector system was tested in *D. melanogaster* white strain using a helper transposase under *piggyBac* regulation (pBΔSac) and a vector marked solely with *D. melanogaster* mini-*white* gene (pB[Dmw]). A mixture of vector and helper plasmids at concentrations of about 600 and about 400 μg/ml, respectively, was injected into about 2,650 embryos from that about 418 larvae hatched with about 283 emerging as adults. (See Table 1 below). The G0 adults were backcrossed to *w*[m] flies in groups totaling about 111. Four of the G0 lines yielded G1 offspring having varying levels of eye pigmentation (Figure 1). One line (F30) was sterile, and one line produced only white eye offspring, and therefore only two of the putative Dm[pBw] transformants were verified. One of these (F13) exhibited eye pigmentation only in females in several succeeding generations, suggesting that the integration caused a sex-linked lethal mutation. Presuming a

fertility rate of about 50% (fertility rates are typically between about 40-60%; see below), an approximate transformation frequency of about 1-3% of fertile G0s was obtained.

[0053] In a second experiment, the pB[Dmw] vector was again tested but with a *piggyBac* transposase helper under *D. melanogaster hsp*70 (Lis et al., 1983 *supra*) promoter regulation (phsp-pBac). A vector/helper mixture, at a concentration of approximately 600/400 µg/ml was injected into about 1,940 embryos, of which about 247 larvae hatched, with about 122 emerging as adults (See Table 1, below). G0 adults were initially backcrossed in a total of about 49 groups to *w*[m] flies, after which they were individually mated to determine fertility. Of the about 98 surviving G0 flies, about 41 yielded offspring resulting in a fertility rate of about 42%. Of the 41 fertile G0 flies, 11 lines produced offspring having varying levels of eye coloration (Figure 1) yielding a transformation frequency of about 26%. The number of G1 offspring from the G0 lines varied considerably, ranging from 1 G1 in lines M11 and F1, to 102 G1 flies in line M13.

[0054] In a third experiment, the phsp-pBac helper was used, but with a *piggyBac* vector including the enhanced green fluorescent protein (gfp) marker gene in addition to the *D.*

melanogaster white gene. This allowed the testing of a new gfp marker construct in transformants that could be primarily identified by white expression. Although expression of wild type GFP under polyubiquitin-nuclear localizing sequence regulation had been tested previously in *D. melanogaster P* transformants (Davis et al., 1995, *supra*), the vector of the present invention improves expression of GFP by using an enhanced GFP (EGFP-1) having a double mutation causing a reported increase in expression of up to about 35-fold (Cormack et al., 1996, *supra*; Yang et al., 1996, *supra*). The variant form is also optimized for mammalian codon usage and polyadenylation, and preliminary tests of the marker construct indicated transient GFP expression in both *Drosophila* embryos and dipteran and lepidopteran cell lines (A.M. Handler and R.A. Harrell, unpublished). The vector construct, pB[Dmw, PUb-nls-EGFP], also allowed evaluation of *piggyBac* transformation with about a 10.0 kb vector, approximately 3.4 kb larger than previous vectors tested, and having about 748 bp of *piggyBac* DNA deleted (previous vectors retained all *piggyBac* DNA). As before, a mixture of about 600 µg/ml vector and about 400 µg/ml helper was injected into about 2147 embryos, of which about 412 larvae hatched, and about 218 emerged as adults (Table 1 below). G0 adults were backcrossed to

w[m] flies in a total of about 90 mating groups, of which about 79 yielded offspring. Although *white*$^{+}$ gene expression (eye pigmentation) was depended upon as the primary marker, G1 larvae and pupae were examined under ultraviolet light for visible GFP expression, and seven of the G0 lines yielded fluorescent G1 larvae and pupae. Interestingly, as shown below in Table 2, upon adult emergence only six of the seven G0 lines yielded G1 offspring with observable eye color pigmentation. While about 70 G1 offspring in total exhibited observable green fluorescence, only about 27 of these flies exhibited a level of eye pigmentation that would have allowed their selection under normal screening procedures. In contrast, all of the G1 flies with eye color pigmentation expressed GFP. Figure 1b shows a Dm[pB*w*, *egfp*] transformant having an orange eye color and GFP fluorescence, with no fluorescence observed in the *w*[m] host. Figure 1c shows another transformant having a white eye phenotype indistinguishable from that in the *w*[m] host strain, but exhibiting an equal, if not greater level of GFP fluorescence compared to the orange eye transformant. Notably, fluorescence is quenched in the eye of the pigmented transformant, while it is easily visible in the white eye transformant. High magnification examination revealed a few pigmented ommatidia in some white eye

G1 flies expressing GFP, though these would not have been normally detected. Based on selection by GFP expression and presuming about 50% fertility, an approximate transformation frequency of about 6-7% of fertile G0 flies is deduced.

[0055] An assessment of vector activity based on germline transformation frequency is a factor of both transposon mobility in the host embryo and levels of genomic position effect suppression of the marker gene, or stated more simply, the ability to visibly identify putative transformants. While position effect variegation and suppression of *white* expression in transformants is well established (Hazelrigg et al., Cell, Volume 64, 1083-1092, 1984; Pirrotta et al., 1985, *supra*), the effect of complete marker suppression on transformation frequencies has not been assessed since such transformants have been only detected fortuitously after molecular analysis. The experiment using both the *white* and GFP markers proved the importance of position effects on marker expression convincingly, since GFP was readily detectable in 70 G1 flies, yet eye pigmentation was apparent in less than 40% of these. Under typical screening procedures these flies would not have been scored as transformants, though pigmentation in a few ommatidia in some flies could be detected at high magnification, and for a

few lines, pigmentation was more apparent in subsequent generations. It is likely that expression of the *white* marker would have been improved by heat shock regulation, but nonetheless, GFP was easily detected in all the non-pigmented transformants, and strongly expressed in some. The influence of modifier genes on position effect variegation is complex, and target genes (or their promoters) are not equivalently affected (Bhadra et al., Genetics, Volume 150, 251-263, 1998). The polyubiquitin-*gfp* gene may be a target of position effect modifiers, but it is clearly less susceptible to suppression relative to *white* in terms of its expressed phenotype in the same chromosomal context. The data suggests that GFP is a more reliable visible marker than *white*, that portends well for its use as a general marker in other insects.

EXAMPLE 3

[0056] Southern hybridization was performed to verify genomic transposition of the *piggyBac* vectors. Approximately 5-10 µg of genomic DNA was digested with indicated restriction enzymes and separated on about 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were labeled with

[^{32}P]-dCTP by random priming (Gibco BRL) according to the manufacturer's specifications. Probe DNA was generated from indicated *piggyBac* restriction fragments (see below) that were separated from p3E1.2, or the entire egfp gene from pEGFP-1 (Clontech) by agarose electrophoresis and gel-elution. Hybridizations were performed in phosphate buffer, approximately pH 7.5; about 1% BSA; about 7% SDS at about 65°C with an initial wash in about 2X SSC; about 0.2% SDS at about room temperature and about two washes in about 1X SSC; about 0.1% SDS at about 55°C for approximately 30 minutes. Autoradiography was performed by exposure of Kodak X-Omat film at about -90° C.

[0057] Genomic transposition of the *piggyBac* vectors was verified by Southern DNA hybridization. The basic strategy was to perform hybridizations to the 5' vector arm using the *piggyBac* *Sph*I-*Hpa*I or *Nsi*I-*Hpa*I fragment as probe, and the 3' vector arm using the *Hpa*I-*Ase*I or *Hpa*I-*Nsi*I fragment as probe. Using probes to both vector arms, internal fragments spanning most of the vector were detected. Hybridizations to the vector arms and adjacent chromosomal sequence indicate their presence in non-plasmid DNA and indicate the number of integrations, while internal hybridizations that yield known fragment sizes confirm vector integrity.

[0058] For pB[Dmw] transformants, genomic DNA was initially digested with *Bgl*II and hybridized to the labeled *Sph-Hpa piggyBac* fragment, that detects both vector arms resulting in two bands for each integration (Figure 2A). Each intact vector integration should result in one band greater than about 0.67 kb for the 5' arm, and one band greater than about 5.9 kb for the 3' arm. Since varying eye color phenotypes among G1 sublines was observed, and in some cases within G1 sublines, sublines having light orange, dark orange, or red eye coloration from the same G1 sublines were selected for hybridization analysis. For example, flies having differing phenotypes from lines M13-39, M19-90, and M19-91 were hybridized separately, but no difference in the number or sites of insertion were apparent. Of all the lines tested, all had single integrations except for two lines having two integrations (M13-39 and M19-91) and one line having three integrations (F14-63). All the lines with multiple integrations had dark orange or red eye color, though several lines with a single integration also shared these phenotypes. Hybridization patterns for the lines tested indicated that for most of the G0 lines, different integrations were transmitted to many of the G1 sibling offspring. For example, the three G1 sublines tested from both the M3 and M5 G0 lines all show different patterns

indicating at least three independent integrations occurring in the two G0 germlines.

[0059] Genomic DNA digested with *Sal*I and hybridized to *Hpa*I-*Ase*I probe yielded single bands greater than about 3.0 kb for each integration, and the number of integrations determined were consistent with the *Sph*I-*Hpa*I hybridizations (Figure 2B). For all samples, *Nsi*I digestion and hybridization to *Nsi*-*Hpa*I and *Hpa*I-*Nsi*I probe yielded only about 1.5 kb and about 4.6 kb bands accounting for about 6.1 kb of the about 6.6 kb vector, indicating the same generally high level of vector integrity for all integrations tested.

[0060] G1 sublines from six G0 lines transformed with the pB[Dm*w*, PUb-nls-EGFP] vector were digested with either *Bgl*II and probed with *Sph*I-*Hpa*I *piggyBac* DNA for 5' vector arm analysis, or digested with *Xho*I and probed with *Hpa*I-*Ase*I *piggyBac* DNA for 3' arm analysis (Figure 3A and 3B). Both hybridizations yielded one band for each sample, indicating single integrations having occurred in each line. *Nsi*I restriction digests with *Nsi*I-*Hpa*I and *Hpa*I-*Nsi*I hybridizations yielded about 0.7 kb and about 0.8 kb bands indicating vector integrity for each integration (data not shown).

[0061] Two G0 lines, M9 and M47, yielded a high proportion of

G1 flies expressing only GFP and white eyes, and line M45 that yielded only white eye transformants. These lines were analyzed by *Pst*I digestion and hybridization to EGFP and *Hpa-Ase*. All lines shared the about 4.4 kb internal vector fragment, with an additional junction fragment from the 3' vector arm and adjacent insertion site chromosomal DNA. The M9 white eye lines all shared the same integration indicated by an about 0.9 kb junction fragment, and similarly the M47 white eye lines all shared the same 5.0 kb junction fragment. The pigmented lines M9-2 and M9-3 had different integrations from each other, and from their white eye sibling lines, and the pigmented lines M47-9 and M47-10 shared the same integration based on an about 4.0 kb junction fragment, but which differs from their white eye siblings. These hybridizations, and that for M45-1, proves that the white eye flies were transformed, and that white expression was likely influenced by differing insertion sites from their pigmented sibling lines.

EXAMPLE 4

[0062] To verify that *piggyBac*-mediated chromosomal transpositions had occurred, insertion sites were isolated by inverse PCR from sublines F1-2, M17-4 and M31-6, all having

single integrations. Inverse PCR was performed as described previously (Handler et al., 1998, *supra;* herein incorporated by reference) using *Hae*III digestions for 5' and 3' junctions and *Msp*I digestion for 3' junctions. After about 4 hours digestion, restriction fragments were circularized by ligation at about 16° C for about 16 hours. PCR was preformed on the circularized fragments by using primer sequences in opposite orientation within the *piggyBac* restriction site and terminus for each junction. For the 5' junction, the forward primer (572F) 5'-TCTTGACCTTGCCACAGAGG-3' (SEQ ID NO 2) and reverse primer (154R) 5'-TGACACTTACCGCATTGACA-3' (SEQ ID NO 3) were used. For the 3' junction the reverse primer (2118R) 5'-GTCAGTCCAGAAACAACTTTGGC-3' (SEQ ID NO 4) and the forward primer (2385F) 5'-CCTCGATATACAGACCGATAAAAACACATG-3' (SEQ ID NO 5) were used. PCR products were separated in low-melting-temperature agarose, and fragments were selected that were longer than the respective restriction site terminus distances and different from those expected from the p3E1.2 based vector and helper plasmids. These products were directly subcloned into ddT vectors (Invitrogen), that were sequenced by using primers to vector sequence proximal to the respective termini. Subcloned PCR products were sequenced and analyzed by alignment using GeneWorks 2.5 software (Oxford

Molecular Group) and subjected to BLAST analysis (Altshul et al., J. Mol. Biol., Volume 215, 403-410, 1990; herein incorporated by reference) to identify genomic insertion site sequences and distinguish them from those in the injected plasmids. For all the integrations both the 5' and 3' junctions yielded the *piggyBac* inverted terminal repeat sequences immediately adjacent to a TTAA sequence (SEQ ID NO 1) and proximal insertion site DNA (Figure 4). The TTAA (SEQ ID NO 1) duplicated target site is characteristic of all *piggyBac* integrations (Elick et al., Genetica, Volume 97, 127-139, 1995) and typically indicates a vector-mediated transposition. The BLAST analysis revealed that the M17-4 integration occurred in a TTAA site within the *cubitus interruptus*-Dominant gene located on chromosome 4 at nucleotide 12,898 (GenBank submission U66884; Ahmed & Podemski, Gene, Volume 197, 367-373, 1997), and the M3106 integration was found to have occurred in a TTAA site within a previously sequenced region of the distal X chromosome (GenBank submission AL09193; Murphy et al, direct submission). Determination of insertions in these previously sequenced sites gives the first direct proof that a *piggyBac* vector does indeed
insert into and duplicates TTAA (SEQ ID NO 1) insertion sites in a eukaryotic genome.

[0063] Two of the three insertion sites that were sequenced were found to be in previously sequenced genomic loci, and as expected, the insertions sites were all TTAA (SEQ ID NO 1) with one of them within the ci^D allele on the fourth chromosome. Many transposons have insertion site preferences, and for at least some, a clear negative bias against specific sites or loci. This has been clearly demonstrated by genomic hotspots and coldspots for *P* integration in *D. melanogaster* (See Engels, In: *Mobile DNA*, D.E. Berg and M.M. Howe, eds., American Society of Microbiology, Washington, D.C., 439-484, 1989), and by differences in preferential integration sites between *hobo* and *P* (Smith et al., Genetics, Volume 135, 1063-1076, 1993). If the TTAA (SEQ ID NO 1) specificity for *piggyBac* integration is not further influenced by proximal sequences, then *piggyBac* transpositions may find use in transposon-mutagenesis and enhancer traps for loci refractory to *P* or *hobo* transpositions in *Drosophila*.

Example 5

[0064] The Caribbean fruit fly, *Anastrepha suspensa*, was transformed with a *piggyBac* vector marked solely with PUb-nls-GFP(pB[PUb-nls-EGFP]) (Figures 5 and 6) using the *hsp70-piggyBac* (phsp-pBac) helper. From injected embryos, 561 surviving G0

adults were intermated in 60 small groups. Four of the G0 groups yielded a total of 57 G1 offspring exhibiting green fluorescence at all stages of development (See Figure 7) and chromosomal vector integrations were verified by Southern hybridization for each G0 group. To test GFP as a genetic marker for field released transgenic flies, the perdurance of GFP expression was assayed in transgenic flies killed by decapitation. Two to three day old *A. suspensa* adults transformed with pB[PUb-nls-EGFP], and wild type non-transformed adults, were decapitated and placed within a plastic box kept outdoors in partial shade. GFP fluorescence was observed daily by digital images taken with a SPOT-1 cooled CCD digital camera (Diagnostic Instruments, Inc.) through a Leica MZ-12 stereozoom microscope. All images were taken at the same magnification and exposure parameters. Figure 10 shows that while GFP fluorescence decreases with time after death, unambiguous detection of GFP is still possible at 28 days after decapitation, with no fluorescence detectable in wild flies (Figure 10). This indicates that the PUb-nls-EGFP marker should be a reliable visible detection system for released transgenic insects, and especially for those captured and killed in field traps with monitoring occurring after extended time periods.

Example 6

[0065] A *piggyBac* vector marked with the Mediterranean fruit fly (*Ceratitis capitata*) white gene cDNA (pB[Ccw]) and the phsp-pBac helper was used to transform the oriental fruit fly (*Bactrocera dorsalis*). Injected G0 embryos from the *B. dorsalis* white eye mutant strain yielded 102 fertile adults, that upon individual backcrossing, yielded three lines of putative transformants with pigmented eyes (Figures 8a-8e). One of these lines produced 119 G1 transformants. Southern DNA hybridization analysis with *piggyBac* and *white* gene probe verified chromosomal integration of the *piggyBac-white* vector in all three lines. In a separate experiment, the *white*/PUb-nls-EGFP marker within pB[Ccw, PUb-nls-EGFP] was introduced into a single *B. dorsalis* transformant line from 17 G0 matings. As in Drosophila, the transformant was selected solely by GFP expression, having undetectable eye coloration. This reaffirms the notion that the polyubiquitin-EGFP marker is significantly more reliable than *white* gene markers.

Example 7

[0066] The PUb-nls-EGFP marker was introduced into the medfly, *Ceratitis capitata*, to further test GFP as a transgenic

selection, and to create GFP-marked strains for testing as a field release marker in medfly SIT. First a *piggyBac* vector marked with PUb-nls-GFP and the medfly *white* gene (pB[Ccw, PUb-nls-EGFP]) was tested, and then the vector solely marked with PUb-nls-GFP pB[PUb-nls-EGFP]) was tested. Both experiments used the *hsp70-piggyBac* (phsp-pBac) helper. Based on GFP fluorescence, the first experiment yielded five transformant lines from 99 fertile G0s (See Figure 9), while the second experiment yielded three transformed lines from 17 fertile G0s. Transformation was verified by Southern hybridization analysis.

EXAMPLE 8

[0067] The plasmid pB[PUbDsRed1] *piggyBac* vector marked with polyubiquitin-regulated DsRed1 (Matz et al., Nat. Biotechnol., Volume 17, 969-973, 1999; herein incorporated by reference) was created by isolating the polyubiquitin promoter (Lee et al., Mol. Cell. Biol., Volume 8, 4727-4735, 1988; herein incorporated by reference) as an *Eco*RI-*Bgl*II fragment from PUbnlsGFP (Davis et al., 1994, *supra;*) and ligating it into the *Eco*RI-*Bgl*II N-terminal cloning site of pDs-Red1-N1 (Clontech, Palo Alto, CA), creating pPUbDsRed1. The polyubiquitin-DsRed 1 gene was isolated as a *Bgl*II-*Not*I fragment that was used to replace the

PUb-nls-EGFP-1 marker cassette within the *Bgl*II-*Not*I site of pB[PUb-nls-EGFP] (Handler and Harrell, Insect Mol. Biol., Volume 8, 449-458, 1999; herein incorporated by reference). Construction of the *hsp*70-regulated transposase helper, phspBac, was as described in Handler and Harrell (*supra*).

[0068] The pB[hsp-GAL4, EGFP] vector was created by ligating the hsp70-GAL4 cassette, from pF89 (Brand et al., Development, Volume 118, 401-415, 1993; herein incorporated by reference) as a *Bgl*II-*Stu*I fragment, into the *Bgl*II and blunted *Bst*BI site of pB[PUb-nls-EGFP]. The pB[UAS-DsRed1,EGFP] vector was created by isolating DsRed1 from pDsRed1-N1 as a *Bam*HI/*Not*I fragment and ligating it into the *Bgl*II/*Not*I sites of pUAST (Brand et al, *supra*) to create pUAS-DsRed1. The UAS-DsRed1 fragment from pUAS-DsRed1 was then isolated as a *Bam*HI fragment and ligated into the *Bgl*II site of pB[PUb-nls-EGFP].

EXAMPLE 9

[0069] Fluorescent protein expression was observed at various developmental stages from transgenic insects having single integrations of DsRed or EGFP as determined by Southern analysis. Fluorescence was observed under a Leica MZ-12 stereozoom fluorescent microscope using a mercury lamp and appropriate

filter sets (Chroma Technology Corp., Brattleboro, VT). For DsRed detection the HQ Texas Red™ set #41004 was used having the following filters: exciter HQ560/55x; dichoric Q595LP; emission HQ645/75m. For EGFP detection the FITC/RSGFP LP Emission set #HQ 41012 was used having the following filters: exciter HQ480/40; dichroic Q505LP; emission HQ510LP. Digital images were obtained with a SPOT-1 cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI) and captured with Adobe Photoshop 4.0 software (Adobe Systems Inc., San Jose, CA). For comparison fo fluorescent protein expression automatic exposures were calculated and images taken for each protein from a transformed adult using the appropriate filter set. These settings were also used as a user-defined exposure for the other protein.

EXAMPLE 10

[0070] Germ-line transformation was tested in the *white* mutant strain, w[m], of *Drosophila melanogaster* with the *piggyBac* vector, pB[PUbDsRed1], having the DsRed1 gene (Figure 11) (Clontech; Matz et al., 1999) regulated by the *D. melanogaster* polyubiquitin promoter. The *hsp70*-regulated *piggyBac* transposase helper was coinjected with the vector. Expression of DsRed1 from the vector construct was tested in preliminary studies by

transient expression in *Drosophila* and *Anastrepha suspensa* embryos after injection with a plasmid containing polyubiquitin-regulated DsRed1. For the transformation experiment the pB[PUbDsRed1] vector was mixed with the phsp-pBac helper at concentrations of 600 µg/ml vector and 400 µg/ml helper and injected into 713 eggs of which 305 larvae hatched. Of these, 191 larvae survived to adulthood, including 101 G0 males and 90 G0 females. The G0 adult progeny were backcrossed to w[m] flies in 81 small groups that included either two G0 males (50 groups) or three G0 females (31 groups). All of the groups yielded viable G1 progeny that were screened as larvae, pupae, and adults for DsRed1 expression using a Texas Red filter set (Figures 12-15). Of the 81 mating groups, 26 groups yielded G1 progeny expressing red fluorescence. Presuming one G0 transformation event per mating group, and 100% fertility, a minimum frequency of transformation in this experiment is 13.6%. Numerous previous transformation experiments have yielded G0 fertility rates of approximately 50%, which would yield a frequency of about 27% which is similar to previous transformations with the *piggyBac* vector in *D. melanogaster*.

EXAMPLE 11

[0071] *D. melanogaster* was transformed with pB[hsp-GAL4,EGFP] and pB[UAS-DsRed1, EGFP] expression vectors as described above in Example 9. The transformed insects were inbred as single pair matings for successive generations until all progeny expressed the EGFP marker and were considered homozygous. Adult flies from the hsp-Gal4 and UAS-DsRed1 lines were intermated and their progeny subjected to heat shock at about 37° C for about one hour at indicated times. DsRed expression was monitored at daily intervals.

[0072] The use of DsRed as a reporter in an EGFP background was first detected in pharate adult pupae one day after two daily heat shocks (two days after the first heat shock). The ability of filter systems for DsRed to effectively block EGFP fluorescence indicates that DsRed can be used as an unambiguous reporter in tissue where EGFP is co-expressed.

[0073] The foregoing detailed description is for the purpose of illustration. Such detail is solely for that purpose and those skilled in the art can make variations without departing from the spirit and scope of the invention.